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(54) Title: TREATMENT OF HUMAN TUMORS BY GENETIC TRANSFORMATION OF HUMAN TUMOR CELLS

(57) Abstract

A method of treating a tumor in a human host wherein tumor cells are transduced *in vivo* with a nucleic acid sequence (DNA or RNA) encoding an agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence encoding the agent. In one embodiment, such treatment is effected by administering to the tumor *in vivo* producer cells transduced with a retroviral vector including a gene encoding a negative selective marker. Once the producer cells are administered to the tumor, the producer cells generate infectious viral particles which infect the tumor cells. Upon subsequent administration of an interaction agent which interacts with the negative selective marker to produce an agent which is toxic to the tumor cells, the transduced tumor cells are killed. In a preferred embodiment, the method is used to treat human brain tumors.

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TREATMENT OF HUMAN TUMORS BY GENETIC TRANSFORMATION OF HUMAN TUMOR CELLS

The present application is a continuation-in-part of and has benefit, for priority purposes, of the filing date of U.S. application serial No. 08/116,669 (filed September 3, 1993) and U.S. application serial No. 07/877,519 (filed May 1, 1992), the contents of both of which are hereby incorporated by reference.

This invention relates to the treatment of human tumors by transforming or transducing human tumor cells with DNA (RNA) encoding an agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of such DNA (RNA) encoding the agent.

Gene transfer has been recognized for some time as a promising avenue to therapies for cancers, among other diseases. The earliest applications of gene transfer for cancer treatment have been indirect approaches focusing on enhancing anti-tumor immune responses. Thus, for instance, attempts have been made to increase the cytotoxicity of immune cells, or to enhance their proliferation.

In one such approach, the gene for tumor necrosis factor (TNF) was inserted into tumor infiltrating

lymphocytes (TIL) in an attempt to use the homing properties of TIL to deliver the toxic gene product preferentially to the tumor in situ. Initiation of this protocol has been difficult, however, because transduced T-cells shut down vector cytokine expression. Rosenberg et al., Human Gene Therapy, 1: 443 (1990).

In another approach, tumor cells have been modified in vitro with cytokine genes and reintroduced into patients in an attempt to immunize the patient to their own cancer. In animal studies, the IL-4 gene was introduced to tumors by Tepper, et al., Cell 57: 503 (1989); the IL-2 gene by Fearon, et al., Cell 60 :397 (1990), and by Gansbacher, et al., J. Exp. Med. 172: 1217 (1990); the interferon-gamma gene by Gansbacher, et al., Cancer Res. 50: 7820 (1990); and TNF gene by Asher, et al., J. Immunol. 146: 3227 (1991). Each of the animal studies demonstrated rejection of genetically altered tumors upon reimplantation, and the mice in these studies were immune to subsequent rechallenge with the same tumor.

These early investigations of the clinical use of gene transfer required that the tumor be excised and TIL or tumor cell lines established in culture which then could be gene-transduced in vitro and subsequently reimplanted into the patient. This approach is limited by the fact that TIL and tumor lines cannot be regrown in vitro from the tumors of all patients and by the necessity to perform ex vivo transduction.

Retroviral vectors currently provide the most efficient means for ex vivo gene transfer in the clinical setting, but their usefulness has been seen as limited because retroviruses stably integrate only in target cells that are actively synthesizing DNA, and integration is a prerequisite to retroviral gene expression.

Cancers consist of actively replicating cells, however, and are often surrounded by quiescent normal

cells. Thus, the above limitation may be exploited as an advantage in treating cancers, because a retroviral vector that carries a therapeutic agent would be integrated and expressed preferentially or exclusively in the cells of the cancerous mass.

In this regard Ezzeddine, et al., New Biologist 3: 608-14 (1991), have reported on the use of retroviral vector-mediated gene transfer in vitro in an attempt to treat tumors. More specifically, a murine retroviral vector was employed to introduce a thymidine kinase gene from herpes simplex virus 1 ("HSV-1 tk gene") into C6 rat glioma-derived cell lines in vitro. Cells which had taken up the retroviral vector were sensitized to the anti-viral agent ganciclovir, and were preferentially killed when exposed to ganciclovir in the medium.

Ezzeddine, et al. were able to use the method to define conditions in vitro for killing essentially all infected cells but not uninfected cells. In addition, C6 cells were introduced subcutaneously into nude mice to form tumors and the tumor-bearing mice were treated with ganciclovir. Ganciclovir inhibited the growth of tumors formed by HSV-1 tk expressing C6 cells, but did not affect tumors formed by HSV-1 tk-negative C6 cells.

Ezzeddine, et al. thus showed that in vitro retroviral gene transfer can be used to sensitize cells to a cytotoxic agent, which can then be used to kill the cells when they are propagated as tumors in nude mice. The authors did not demonstrate any practical way to introduce an HSV-1 tk gene into tumor cells in situ, however. Ezzeddine, et al. also did not show how to eradicate all neoplastic cells, a prerequisite for tumor remission, when less than all cells in the tumor would take up a tk gene, express the gene at a level sufficient to assure toxicity and, as a consequence, be killed by exposure to ganciclovir.

Short, et al., J. Neurosci. Res. 27: 427-33 (1990), have described the delivery of genes to tumor cells by means of grafting a retroviral vector-packaging cell line into a tumor. The packaging cell line produced a replication-defective retroviral vector in which the MoMLV LTR promoter-operator was used to drive expression of ß-galactosidase, which served as a marker of retroviral vector propagation. When the packaging cell line was grafted into a tumor, ß-galactosidase expression in situ was seen only in packaging cells and in proliferating tumor cells, not in normal tissue.

Despite the apparent preference for tumor cells, propagation of the retroviral vector from producer cells to tumor cells was relatively inefficient, according to Short, et al., and only a fraction of the cells in the tumor were infected. Furthermore, practically no galactosidase expression was observed when cell-free retroviral vector particles were introduced to a tumor directly rather than in a packaging cell line. Short, et al. opined that a packaging cell line might be used to deliver a "killer" or "suppressor" gene to tumor cells, but observed an efficiency of infection far below what would be required for therapeutic utility based on direct gene transduction into all the cells of a tumor.

Malignant brain tumors are responsible for significant morbidity and mortality in both pediatric and adult populations. These common tumors present an enormous therapeutic challenge due to their poor outcome despite radical surgery, high dose radiotherapy and chemotherapy. Survival of patients from the time of diagnosis is measured in months and recurrence after treatment is associated with a life expectancy of weeks.

Culver, et al., Science, Vol. 256, pgs. 1550-1552 (June 12, 1992) and Ram, et al., Cancer Research, Vol. 53, pgs. 83-88 (January 1, 1993), disclose the administration

by intratumoral injection of fibroblasts producing a retroviral vector which includes the Herpes Simplex thymidine kinase gene to rats with cerebral glioma. After the rats were given the producer cells, they were given ganciclovir.

PCT Application No. W093/04167, published March 4, 1993, discloses a method for transferring therapeutic genes to brain tumor cells in order to kill the cells. In such method, a retrovirus containing a selectable marker and at least one gene required for its replication is introduced into producer cells such that integration of the proviral DNA corresponding to the retrovirus into the genome of the producer cell results in the generation of a modified retrovirus wherein at least one of the genes required for replication of the retrovirus is replaced by the therapeutic gene or genes. Producer cells then are selected in which the modified retrovirus is incorporated as part of the genome of the producer cells. The producer cells then are grafted in proximity to the dividing tumor cells in order to infect the tumor cell with the modified retrovirus, thereby transferring the therapeutic gene or genes to the tumor cells. The cells then are killed by administering a substance that is metabolized by the therapeutic gene transferred to the tumor cells into a metabolite that kills the cells. The therapeutic gene may be the Herpes Simplex thymidine kinase gene, and the substance which is metabolized by Herpes Simplex thymidine kinase to kill the tumor cells may be ganciclovir or acyclovir. The cited PCT application shows only (i) that a replication-defective retrovirus which carried an HSV tk gene and a G418 resistance gene could be transduced stably, via G418 selection, into a glioma cell line in vitro; (ii) that the viral tk gene in the transformed cells rendered them about 20-fold more sensitive to ganciclovir than control glioma cells; and (iii) that some glioma tumor

cells which formed tumors when implanted in rat brains also expressed a β-galactosidase marker when the tumors were injected with a producer cell line which produced a retroviral vector with the marker gene. The vector in the described experiments did not carry a tk gene, and there was no systemic administration of a chemotherapeutic agent. Thus, the PCT application in question does not show that tumor cells can be rendered sensitive in vivo to any such agent.

In accordance with an aspect of the present invention, there is provided a method of treating a tumor in a human host, preferably a brain tumor. The method comprises transducing tumor cells in vivo with a nucleic acid (DNA or RNA) sequence encoding an agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence encoding the agent.

The nucleic acid sequence which encodes the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence is contained in an appropriate expression vehicle which transduces the tumor cells. Such expression vectors include, but are not limited to, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial vectors), and viral vectors.

In one embodiment, the expression vector is a viral vector. Viral vectors which may be employed include, but are not limited to, retroviral vectors, adenovirus vectors, adeno-associated virus vectors, and Herpes virus vectors. Preferably, the viral vector is a retroviral vector.

In a preferred embodiment, a packaging cell line is transduced with a viral vector containing the nucleic acid sequence encoding the agent which provides for the inhibition, prevention, or destruction of the tumor cells upon expression of the nucleic acid sequence encoding the

agent to form a producer cell line including the viral vector. The producer cells then are administered to the tumor, whereby the producer cells generate viral particles capable of transducing the tumor cells.

In a preferred embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. Preferably, the retroviral vector is an infectious but non-replication competent retrovirus. However, replication competent retroviruses may also be used.

Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

These new genes have been incorporated into the proviral backbone in several general ways. The most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells.

Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter.

Efforts have been directed at minimizing the viral component of the viral backbone, largely in an effort to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

In one embodiment, the retroviral vector may be one of a series of vectors described in Bender, et al., J. Virol. 61:1639-1649 (1987), based on the N2 vector (Armentano, et al., J. Virol., 61:1647-1650) containing a series of deletions and substitutions to reduce to an absolute minimum the homology between the vector and packaging systems. These changes have also reduced the likelihood that viral proteins would be expressed. In the first of these vectors, LNL-XHC, there was altered, by site-directed mutagenesis, the natural ATG start codon of gag to TAG, thereby eliminating unintended protein synthesis from that point. In Moloney murine leukemia virus (MoMuLV), 5' to the authentic gag start, an open reading frame exists which permits expression of another glycosylated protein ($pPr80^{gag}$). Moloney murine sarcoma virus (MoMuSV) has alterations in this 5' region, including a frameshift and loss of glycosylation sites, which obviate potential expression of the amino terminus of $pPr80^{gag}$. Therefore, the vector LNL6 was made, which incorporated both the altered ATG of LNL-XHC and the 5' portion of MoMuSV. The 5' structure of the LN vector series thus eliminates the possibility of expression of retroviral reading frames, with the subsequent production of viral antigens in genetically transduced target cells. In a final alteration

to reduce overlap with packaging-defective helper virus, Miller has eliminated extra env sequences immediately preceding the 3' LTR in the LN vector (Miller, et al., Biotechniques, 7:980-990, 1989).

The paramount need that must be satisfied by any gene transfer system for its application to gene therapy is safety. Safety is derived from the combination of vector genome structure together with the packaging system that is utilized for production of the infectious vector. Miller, et al. have developed the combination of the pPAM3 plasmid (the packaging-defective helper genome) for expression of retroviral structural proteins together with the LN vector series to make a vector packaging system where the generation of recombinant wild-type retrovirus is reduced to a minimum through the elimination of nearly all sites of recombination between the vector genome and the packaging-defective helper genome (i.e. LN with pPAM3).

In one embodiment, the retroviral vector may be a Moloney Murine Leukemia Virus of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al. (1987) and Miller, et al. (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragments or truncations thereof, are not expressed.

In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred

embodiment, the retroviral vector includes each of these cloning sites. Such vectors are further described in U.S. Patent Application Serial No. 919,062, filed July 23, 1992, and incorporated herein by reference.

When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited

to, the histone, pol III, and ß-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vector then is employed to transduce a packaging cell line to form a producer cell line. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Ψ-2, Ψ-AM, PA12, T19-14X, VT-19-17-H2, Ψ CRE, Ψ CRIP, GP+E-86, GP+envAm12, and DAN cell lines. The vector containing the nucleic acid sequence encoding the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence encoding the agent may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation.

The producer cells then are administered directly to or adjacent to the tumor in an amount effective to inhibit, prevent, or destroy the growth of the tumor. In general, the producer cells are administered in an amount of at least 2.5×10^8 cells per cc of tumor. In general, the amount of cells administered does not exceed 4×10^8 cells per cc of tumor; however, greater amounts may be used. The exact amount of producer cells to be administered is dependent upon various factors, including but not limited to, the type of the tumor and the size of the tumor.

In general, the producer cells are administered directly to or adjacent to the tumor by injection. For example, the cells may be administered by use of a CT or MRI guided stereotaxic system which permits representation of a tumor mass on a two dimensional implantation grid, such as one containing 89 holes. The system can provide

the xact coordinates, positions and injection tracts to optimize the distribution of producer cells into a tumor mass.

The producer cells are administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier such as, for example, a saline solution or a buffer solution or other isomolar aqueous solution.

Upon administration of the producer cells to the tumor, the producer cells generate viral particles. The viral particles then transduce the surrounding tumor cells. Because tumor cells, and in particular cancerous tumor cells, in general are actively replicating cells, the retroviral particle would be integrated into and expressed preferentially or exclusively in the tumor cells as opposed to normal cells.

Tumors which may be treated in accordance with the present invention include malignant and non-malignant tumors.

Malignant (including primary and metastatic) tumors which may be treated include, but are not limited to, those occurring in the adrenal glands; bladder; bone; breast; cervix; endocrine glands (including thyroid glands, the pituitary gland, and the pancreas); colon; rectum; heart; hematopoietic tissue; kidney; liver; lung; muscle; nervous system; brain; eye; oral cavity; pharynx; larynx; ovaries; penis; prostate; skin (including melanoma); testicles; thymus; and uterus.

In accordance with the present invention, the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of such agent is a negative selective marker; i.e., a material which in combination with a chemotherapeutic or interaction agent inhibits, prevents or destroys the growth of the tumor cells.

Thus, upon transduction of the tumor cells with the negative selective marker, an interaction agent is administered to the human host. The interaction agent interacts with the negative selective marker in order to prevent, inhibit, or destroy the growth of the tumor cells.

Negative selective markers which may be employed include, but are not limited to, thymidine kinase, such as Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase; and cytosine deaminase.

In one embodiment, the negative selective marker is a viral thymidine kinase selected from the group consisting of Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase. When such viral thymidine kinases are employed, the interaction or chemotherapeutic agent preferably is a nucleoside analogue, for example, one selected from the group consisting of ganciclovir, acyclovir, and 1-2-deoxy-2-fluoro-β-D-arabinofuranosil-5-iodouracil (FIAU). Such interaction agents are utilized efficiently by the viral thymidine kinases as substrates, and such interaction agents thus are incorporated lethally into the DNA of the tumor cells expressing the viral thymidine kinases, thereby resulting in the death of the tumor cells.

In another embodiment, the negative selective marker is cytosine deaminase. When cytosine deaminase is the negative selective marker, a preferred interaction agent is 5-fluorocytosine. Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is highly cytotoxic. Thus, the tumor cells which express the cytosine deaminase gene convert the 5-fluorocytosine to 5-fluorouracil and are killed.

The interaction agent is administered in an amount effective to inhibit, prevent, or destroy the growth of the transduced tumor cells. For example, the interaction agent

may be administered in an amount from 5 mg to 10 mg/kg of body weight, depending on overall toxicity to a patient. The interaction agent preferably is administered systemically, such as, for example, by intravenous administration, by parenteral administration, by intraperitoneal administration, or by intramuscular administration.

When producer cells or other expression media including a negative selective marker are administered to a tumor in vivo, a "bystander effect" may result, i.e., tumor cells which were not originally transduced with the nucleic acid sequence encoding the negative selective marker may be killed upon administration of the interaction agent. Although the scope of the present invention is not intended to be limited to any theoretical reasoning, the transformed tumor cells may be producing a diffusible form of the negative selective marker that either acts extracellularly upon the interaction agent, or is taken up by adjacent, non-transformed tumor cells, which then become susceptible to the action of the interaction agent. It also is possible that one or both of the negative selective marker and the interaction agent are communicated between tumor cells.

In a preferred embodiment, a packaging cell line is transduced with a retroviral vector, such as those hereinabove described, which includes the Herpes Simplex Virus thymidine kinase gene. The transduced packaging cells (producer cells) are administered in vivo to the tumor in an acceptable pharmaceutical carrier and in an amount effective to inhibit, prevent, or destroy the growth of the tumor. Upon administration of the producer cells to the tumor, the producer cells generate viral particles including a gene encoding the negative selective marker. Such viral particles transduce the adjacent tumor cells. The human host then is given an agent such as ganciclovir,

acyclovir, or 1-2-deoxy-2-fluoro-β- D-arabinofuranosil-5-iodouracil (FIAU), which interacts with the Herpes Simplex Virus thymidine kinase to kill the transduced tumor cells. As hereinabove mentioned, a "bystander effect" may also occur, whereby non-transduced tumor cells also may be killed as well.

In a preferred embodiment of the present invention, a coordinated system of diagnostic imaging, computer-image analysis and stereotaxic surgical manipulations is employed to administer a producer cell to a tumor with the least damage to the patient and with maximum therapeutic effect. As described in greater detail below, by means of this system a set of multiple, parallel microinjection trajectories can be determined, and producer cells are deposited at sites along the trajectories. In this manner, the producer cells are more evenly distributed, preferably to effect an equi-volumetric, homogeneous distribution of the producer cells in the tumor.

In accordance with this aspect of the invention, data for a three-dimensional image of the tumor in situ first are obtained, preferably via a diagnostic imaging technique such as computerized tomography (CT) or magnetic resonance (MR) imaging. An image employed in this context should contain markers, called "fiducials," which appear as spots on the image and which relate in a precise manner to the anatomy of the individual patient.

Thus, the fiducials provide a means for precise translation of co-ordinates in the images into co-ordinates on a stereotaxic instrument for neuroinjection into the individual patient. The use of fiducials for this purpose is a well-known expediency in neurosurgical technique.

The data from the CT or MR image preferably are translated for use by a software program that facilitates interactive analysis to determine sites and trajectories for microinjecting a producer cell, such as retroviral

producer cells, into the tumor. The analysis can be carried out on a high-end graphics workstation or a more powerful computer to permit real-time interactive development of the microinjection plan.

A variety of commercially available software and hardware can be modified for use in accordance with this aspect of the present invention. Software that has been developed specifically for planning stereotaxically guided procedures may be adapted most readily for this purpose. Exemplary of such software is the stereotaxic planning system commercialized by BrainLAB (Munich, Germany), implemented in the BrainSCAN software. The BrainSCAN software provides for manipulation of three-dimensional tumor images acquired, for example by CT or MR imaging. The software provides convenient utilities for viewing images, identifying and visualizing a tumor mass in an organ, calculating a volume, calculating a physical center, plotting microinjection trajectories, displaying the passage of trajectories through a tumor and, by the fiducials in an image, translating co-ordinates in an image into co-ordinates for use on a stereotaxic instrument. Accordingly, the software represents a powerful tool for a physician's identifying the most effective set of microinjection trajectories. Its use in planning a therapeutic procedure in accordance with the present invention is illustrated below.

In current practice, the CT or MR data provide two-dimensional, sectional views of the imaged area of the patient which combined computationally, yield a three-dimensional view. The tumor is identified in the two-dimensional views by the physician, who outlines the contour of the tumor(s) using a light pen or similar device. After entering the therapeutic targets into the image analysis program in this way, the physician can use

the software utilities to view and assess the efficacy of different neurological approaches.

The use of such a program, can be understood by illustrative reference to a preferred embodiment of the present invention which entails homogeneously distributing a retroviral producer cell line in a brain tumor by multiple, parallel microinjections. In this embodiment the BrainSCAN software has been adapted to depict a grid which has been developed for guiding the microinjections. The physician in effect can place the grid anywhere on the surface of the brain.

The software, on command, will display a microinjection trajectory for each needle guide in the grid, which accommodates many needles. The program illuminates trajectories that pass through a tumor and shows their path. The software summarizes which needles will pass through the tumor and the percentage of the tumor that will be covered by the intersecting trajectories. By moving the grid to different points on the surface of the brain, the physician can observe the trajectories and assess the summary data to develop an effective plan for neurosurgery.

Pursuant to the present invention, a paramount consideration in this regard is achieving a homogeneous distribution of retroviral producer cells throughout the tumor. For this purpose, it has been found that the sites of cell deposition should be centered as much as possible on roughly equal volumes of the tumor. That is, the distribution of the producer cells should be equivolumetric, insofar as practical for the shape and accessibility of the tumor mass(es). Chosen to achieve this end, therefore, will be (i) the entry position, exposed by craniotomy in accordance with the present invention, where the grid will be placed on the brain; (ii) a set of parallel trajectories and, correspondingly, of

needle positions in the grid; and (iii) the deposition sites along each trajectory.

A number of additional factors also will be weighed in choosing the entry point, the needle positions and the deposition sites. For instance, the entry site should be convenient to the surgical procedure, which must expose the brain for the multiple injections. In the procedure developed for brain tumors, a craniotomy is performed and the entry point is assessed for convenience in relation to the surgical procedure. The trajectories also are chosen to avoid blood vessels. Another objective in planning the surgical procedure is to avoid passing a needle through any area particularly sensitive to mechanical damage. Finally, the grid placement and the trajectories may be chosen to provide the least invasive procedure.

Once the grid placement, the trajectories, and the deposition sites are chosen, the program provides a listing of stereotaxic coordinates for carrying out the microinjection. The co-ordinates correspond exactly to device markings that are used in the surgery. Included in the listing are the depths in each microinjection trajectory for depositing cells. The procedure may be executed according to a plan developed in this manner, using well-known neurosurgical procedures, as exemplified below.

This methodology contemplates trajectories and deposition sites that are more closely spaced than is characteristic of conventional neurosurgical techniques. In addition, the production of multiple, parallel tracks along microinjection trajectories determined as described above requires, for properly positioning the needles, exposing a relatively large area of the brain by craniotomy. In addition, the grid for guiding the needles in a particularly preferred embodiment of the present

invention, is placed directly onto the surface of the exposed brain.

A specialized stereotaxic device, one of several particularly preferred embodiments of this aspect of the invention, shown in Figure #9, has been developed specifically to accommodate the foregoing requirements. Figure 9 is a photograph which shows a typical stereotaxic instrument for neurosurgery equipped with a grid system for guiding needles along multiple parallel microinjection trajectories. Surgical devices, including needles, can be seen in their parallel paths in the grid. The depicted device provides a plurality of closely spaced guides for microinjection. The device provides 89 guide holds with a center-to-center spacing of 3mm. As shown, the guides are attached to a stereotaxic instrument which permits precise positioning of the injection needles to follow the trajectories determined during by image analysis of the tumor, described above.

Otherwise, the injection may be carried out using commercially available equipment. The needles and syringes may be those generally employed in neurosurgery. Narrow gauge needles are preferred. Standard syringes may be employed, such as 100 microliter Hamilton syringes. The needle may be inserted into the brain manually. Delivery of fluid also may be controlled manually directly by the syringe plunger.

In delivering the producer cells into the brain, preferably a needle first is inserted along its trajectory to the maximum extent planned and cells are there deposited. Then the needle is withdrawn incrementally, with stops made to deposit cells at predetermined points along the path. In this regard it has been found practical to move the needle in increments of millimeters. The same procedure is used for each trajectory until all the planned depositions have been made.

The spacing of trajectories, the distribution of injection sites along the trajectories, and the volume and concentration of producer cells which will be optimal for different tumors, distinguished by type, stage, site, patient characteristics and the like, and for different vectors and interaction agents, ultimately will be determined empirically, i.e., by reference to therapeutic efficacy. Thus, treatment experience will be incorporated into the foregoing procedures to provide more productive therapeutic designs against an increasingly wider variety of human tumors.

The method of the present invention is particularly useful when the targeted tumor is in a tissue made up of cells which are relatively quiescent mitotically, such as liver, skin, bone, brain muscle, bladder, prostate, kidney, adrenal, pancreas, heart, blood vessel and thyroid tissues, among others. The inventive approach also should be useful against tumors located in the subarachnoid space, in the peritoneum, and in the pleural cavity.

Particularly preferred targets are brain tumors, which display several features making them especially susceptible to treatment in accordance with the present invention. Neurons and most other stationary cells in the brain are quiescent and do not regularly synthesize DNA. Vascular endothelial cells in the brain may be cycling at a low rate, but among those most likely to be in cycle would be cells responding to angiogenesis promoting signals often localized in the vicinity of a tumor. Such vessels would most likely be part of the blood supply of the tumor and therefore their destruction would also be desirable. Within the brain, therefore, the principal mitotically active cells would be tumor cells or cells necessary for its support. Accordingly, retroviral vectors introduced into the brain principally will integrate into and affect

only tumor cells or cells associated with tumor vascularization.

Brain tumors often are localized and yet are often inoperable because of their location in relationship to adjacent critical structures. Accordingly, a technique within the present invention, whereby delivery of a toxic product to the tumor is effected without surgical resection, is very useful. Another advantage of targeting brain tumors in the present invention is that the brain is an immunologically privileged site and, thus, may permit retroviral vector-producer cells which are histoincompatible to persist for a significant period without immunologic rejection.

Direct injection of the producer cells also minimizes undesirable propagation of the virus in the body, especially when replication-competent retroviral vectors are used. Because most cells of the body express receptors for amphotropic retroviral vectors, any vector particle which escapes from the local environment of the tumor should immediately bind to another cell. Most cells are not in cycle, however, and therefore will not integrate the genes carried by the vector and will not express any genes which it contains. Thus, the proportion of potential target cells which are in cycle at the time of exposure will be small, and systemic toxic effects on normal tissues will be minimized.

An alternative and preferred embodiment of the present invention employs surgical resection and implantation of a reservoir (sometimes hereinafter referred to as an "Ommaya Reservoir") into the tumor bed of the brain and thereby provides an access port to the brain for introduction of producer cells of the type hereinabove described. The cells are injected through the overlying skin and into the reservoir.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Gene Therapy for the Treatment of Brain Tumors Using Intratumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir

Brain tumors are a major cause of morbidity and mortality in the population. New brain tumors develop in approximately 35,000 adult Americans each year. They comprise the third leading cause of death from cancer in persons 15 to 34 years of age. Mahaley, et al., J. Neurosurg., Vol. 71, pgs. 826-836 (1989). Recent evidence indicates that the prevalence of primary brain tumors is increasing, especially in the elderly. Saleman, et al., in Apuzzo, ed., Malignant Cerebral Glioma, Park Ridge, Ill., Association of Neurological Surgeons, pgs. 95-110 (1990).

The astroglial brain tumors, including the highly malignant glioblastoma multiforme (GBM), are the most common primary brain tumors. Despite aggressive therapy which includes surgical removal of the tumor and post-operative high dose radiation, the prognosis of patients with GBM is very grim with a median survival of 9 to 10 months. Ammirati, et al., Neurosurgery, Vol. 21, pgs. 607-614 (1989). Although controversial, it appears that neither the quality nor time of survival is significantly improved when chemotherapy is added to surgery and radiation. Walker, et al., J. Neurosurg., Vol. 49, pgs. 333-343 (1978). When glioblastoma multiform recurs, there is 100% mortality within weeks to a few months. In one study, a mean survival of only 36 weeks was found in patients with recurrent GMB who underwent a second operation. Unfortunately, a reasonable quality of life in those patients was limited to 10 weeks following the

diagnosis of recurrent GMB. Harsh, et al., Neurosurgery, Vol. 21, pgs. 615-621 (1987).

Cerebral metastases are a frequent complication of systemic cancer occurring in 20 to 30 percent of patients with cancer, Cairncross, et al., in Walker, ed., Cancer Treatment and Research, Vol. 12, pgs. 341-377, Boston, Martinus Nighoff (1983) (there are 1.1 million new cases of cancer per year in the United States). In 50% of patients, the metastatic disease is localized to the central nervous system. Dellatre, et al., Arch. Neurol., Vol. 45, pgs. 741-744 (1988). A subset of patients may even be cured of their primary cancers only to succumb to the isolated metastatic disease in the brain. Surgery, combined with radiation therapy, is the treatment of choice for a single focus of brain metastasis that is surgically accessible. Median survival using the bimodality therapy (surgery and radiotherapy) reaches 40 weeks. In most patients with metastatic disease to the brain, multiplicity of the lesions, or their inaccessibility, prohibits surgical intervention and limits therapy to radiation alone with a median survival of about 15 weeks. Patchell, et al., N. Engl. J. Med., Vol. 322, pgs. 494-500 (1990).

The central nervous system has several advantages of safety and efficacy for in vivo gene transfer. First, retroviral vectors only integrate and therefore express vector genes in proliferating cells. In the brain, the tumor is the most mitotically active cell, with only macrophage-derived cells, blood cells and endothelial cells at minimal risk. Therefore, the possibility of specific transduction of the tumor is enhanced. Second, the brain is a partially immunologic privileged site, which should allow a somewhat longer survival of the xenogeneic murine cells in the brain and a greater transduction frequency of the growing tumor. A special feature of human gliomas is their ability to depress local immunity. This is thought

to be secondary to down regulation of IL-2 secretion and diminished expression of high affinity IL-2 receptors on T-lymphocytes. Roszman, Immunol. Today, Vol. 12, pgs. 370-374 (1991). The murine cells should survive longer allowing for the transduction of greater numbers of tumor cells. However, this period of survival will be limited since all cells that integrate and express Herpes Simplex Thymidine Kinase will be destroyed by the ganciclovir.

A. Construction of pG1TkSvNa

The following describes the construction of pG1TkSvNa, a schematic of which is shown in Figure 6. This vector contains the Thymidine Kinase (hTK) gene from herpes simplex virus I regulated by the retroviral promoter and the bacterial gene, neomycin phosphotransferase (Neo^R) driven by an SV40 promoter. The hTK gene confers sensitivity to the DNA analogs acyclovir and ganciclovir, while the Neo^R gene product confer resistance to the neomycin analogue, G418.

To make pGITkSvNa, a three step cloning strategy was used. First, the herpes simplex thymidine kinase gene (Tk) was cloned into the G1 plasmid backbone to produce pG1Tk. Second, the Neo^R gene (Na) was cloned into the plasmid pSvBg to make pSvNa. Finally, SvNa was excised from pSvNa and ligated into pG1Tk to produce pG1TkSvNa.

Plasmid pG1TkSvNa was derived from plasmid PG1 (Figure 3). Plasmid pG1 was constructed from pLNSX (Palmer, et al., Blood, Vol. 73, pgs. 438-445. The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6kb EcoRI fragment, containing the 5' Moloney Murine Sarcoma Virus (MoMuSV) LTR, and the 3.0kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used

to generate the vector plasmid pG1 (Figure 3) by the insertion of the 1.6kB EcoRI fragment containing the 5′ LTR into the unique EcoRI site of pGO. Thus, pG1 (Figure 3) consists of a retroviral vector backbone composed of a 5′ portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender, et al. 1987), a 54 base pair multiple cloning site (MCS) containing, from 5′ to 3′ the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindII, ApaI, and ClaI and a 3′ portion of MoMuLV from base pairs 7764 to 7813 (numbered as described (Van Beveren, et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985) (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo^r gene, the ß-galactosidase gene, the hygromycin^r gene, and the SV40 promoter.

To construct pBg (Figure 4) the 3.0 kb BamHI/EcoRI lacZ fragment that encodes β-galactosidase was isolated from pMC1871 (Pharmacia). This fragment lacks the extreme 5′ and 3′ ends of the β-galactosidase open reading frame. Linkers that would restore the complete lacZ open reading frame and add restriction sites to each end of the lacZ gene were synthesized and ligated to the BamHI/EcoRI lacZ fragment. The structure of the 5′ linker was as follows: 5′ - 1/2 NdeI - SphI - NotI - SnaBI - SalI - SacII - AccI - NruI - BgIII - III 27 bp ribosomal binding signal - Kozak consensus sequence/NcoI - first 21 bp of the lacZ open reading frame - 1/2 BamHI - 3′. The structure of the 3′ linker was as follows: 5′ - 1/2 mutated EcoRI - last 55 bp of the lacZ open reading frame - XhoI - HindIII - SmaI - 1/2 EcoRI - 3′. The restriction sites in the linkers were chosen because they are not present in the neomycin resistance gene, the β-galactosidase gene, the hygromycin resistance gene, or the SV40 promoter. The 27 bp ribosomal binding signal was included in the 5′ linker

because it is believed to enhance mRNA stability (Hagenbuchle, et al., Cell 13:551-563, 1978 and Lawrence and Jackson, J. Mol. Biol. 162:317-334, 1982). The Kozak consensus sequence (5'-GCCGCCACCATGG-3') has been shown to signal initiation of mRNA translation (Kozak, Nucl.Acids Res. 12:857-872, 1984). The Kozak consensus sequence includes the NcoI site that marks the ATG translation initiation codon.

pBR322 (Bolivar et al. Gene 2:95, 1977) was digested with NdeI and EcoRI and the 2.1 kb fragment that contains the ampicillin resistance gene and the bacterial origin of replication was isolated. The ligated 5' linker - lacZ - 3' linker DNA described above was ligated to the pBR322 NdeI/EcoRI vector to generate pBg. pBg has utility as a shuttle plasmid because the lacZ gene can be excised and another gene inserted into any of the restriction sites that are present at the 5' and 3' ends of the lacZ gene. Because these restriction sites are reiterated in the pG1 plasmid, the lacZ gene or genes that replace it in the shuttle plasmid construct can easily be moved into pG1.

A 1.74 kB Bg1II/PvuII fragment containing the Herpes Simplex Virus Type I thymidise kinase gene (GenBank accession no. V00467, incorporated herein by reference) was excised from the pX1 plasmid (Huberman, et al., Exptl. Cell Res. Vol. 153, pgs 347-362 (1984) incorporated herein by reference), blunted with the large (Klenow) fragment of DNA polymerase I, and inserted into the unique SnaBI site in the pG1 multiple cloning site, to form plasmid pG1TK. (Figure 5).

A 339 bp PvuII/HindIII SV40 early promoter fragment obtained from the plasmid pSV2Neo (Southern et al, Journal of Molecular and Applied Genetics 1:327-341(1982)) was then inserted into pBg in the unique NruI site to generate the plasmid pSvBg (Figure 5). The pSvBg plasmid was digested

with BglII/XhoI to remove the lacZ gene, and the ends were made blunt using the Klenow fragment. An 852 bp EcoRI/AsuII fragment containing the coding sequence of the neomycin resistance gene was removed from pN2 (Armentano, et al., J. Virol., Vol. 61, pgs. 1647-1650 (1987)), blunted with Klenow fragment and ligated into the 2.5 kb blunted BglII/XhoI fragment generated hereinabove, resulting in pSvNa. The SV40 promoter/neomycin resistance gene cassette was then removed from pSvNa as a 1191bp SalI/HindIII fragment. The pG1Tk plasmid was then digested with SalI/HindIII and ligated with the SV40/neo^r fragment to generate pG1TkSvNa. (Figure 6).

B. Generation of Producer Cell Line

A producer cell line was made from vector plasmid and packaging cells. The PA317/G1TkSvNa producer cell was made by the same techniques used to make previous clinically relevant retroviral vector producer cell lines. The vector plasmid pG1TkSvNa DNA was transfected into a ecotropic packaging cell line, PE501. Supernatant from the PE501 transfected cells was then used to transinfect the amphotropic packaging cell line (PA317). Clones of transinfected producer cells were then grown in G418 containing medium to select clones that contain the Neo^R gene. The clones were then titered for retroviral vector production. Several clones were then selected for further testing and finally a clone was selected for clinical use.

5 x 10^5 PE501 cells (Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989), incorporated herein by reference) were plated in 100 mm dishes with 10 ml high glucose Dulbecco's Modified Essential Medium (DMEM) growth medium supplemented with 10% fetal bovine serum (HGD10) per dish. The cells were incubated at 37°C, in 5% CO_2/air overnight.

The plasmid pG1TKSvNa then was transfected into PE501 cells by $CaPO_4$ precipitation using 50 μg of DNA by the following procedure.

50 μg of DNA, 50 μl 10 x Ca Cl_2, and 450μl of sterile H_2O was mixed in a 15 ml polypropylene tube to yield a 0.25M Ca Cl_2 solution containing 50 μg DNA, 0.5 ml 2x BBS (containing 50 mM N-N-bis- (2-hydroxyethyl)- 2-aminoethane-sulfonic acid, 280 mM Na Cl, 1.5 mMNa_2 HPO_4, and 50 mM Hepes, pH6.95). The DNA solution then was left at room temperature for about 20 minutes to 1 hour. The dishes then were incubated at 35°C in a 3% CO_2 atmosphere overnight.

A culture dish(es) with optimum precipitate following the overnight incubation then was (were) selected. The dish(es) then was (were) washed again with PBS to remove the salt and the salt solution. 10 ml of HGD10 medium then was added to the dish(es), and the dish(es) incubated at 37°C in a 5% CO_2 atmosphere for about 48 hrs.

After 48 hours supernatant was collected from the transfected cells. The dish(es) then was (were) rinsed with 5 ml PBS. The PBS then was removed, and cells were removed with trypsin-EDTA. Serial dilutions of the cells were then inoculated to six 100 mm dishes in medium containing HGD10 and 0.8 mg/ml G418.

The six plates of cells were examined daily. The medium was changed as needed to remove dead cells. Live cells or colonies were allowed to grow to a size such that the colonies are large enough to clone (i.e., the colonies are visible to the naked eye). PE501 supernatants from such colonies of PE501 cells were collected in volumes of from about 5 to 10 ml, placed in cryotubes, and frozen in liquid nitrogen at -70°C.

PA317 cells (Miller et al. Mol. Cell. Biol. 6:2895-2902 (1986)) then were plated at a density of 5 x 10^4 cells per 100 mm plate on Dulbecco's Modified Essential Medium

(DMEM) including 4.5 g/l glucose, glutamine supplement, and 10% fetal bovine serum (FBS).

The PE501 supernatant then was thawed, and 8 μg/ml of polybrene was added to the supernatant. The medium was aspirated from the plates of PA317 cells, and 7 to 8 ml of viral supernatant was added and incubated overnight.

The PE501 supernatant then was removed and the cells refed approximately 18-20 hours with fresh 10% FBS. One day later, the medium was changed to 10% FBS and G418 (800 μg/ml). The plate then was monitored, and the medium was changed to fresh 10% FBS and G418 to eliminate dying or dead cells as necessary. The plate was monitored for at least 10 to 14 days for the appearance of G418 resistant colonies.

The cells were tyrpsinized and incubated into wells in a six well dish in 5 ml of HGD10 plus 1x hypoxanthine aminopterin thymidine (HAT).

If the clones grew to confluency, they were trypsinized and incubated in a 100 ml dish. As a clone in the 100 ml dish approached confluency, its amphotropic vector-containing supernatant was removed and centrifuged at 1,200 to 1,500 rpm for 5 minutes to pellet out cells.

Supernatants were aliquoted into six cryovials (1 ml/vial) and stored in liquid nitrogen. 5 ml of PBS was added to the dish, the cells were rinsed, and refed with HGD10 and frozen in 1 ml aliquots with 10% DMSO in liquid nitrogen. The different clones were monitored to determine the one with the highest titer of retroviral vector. The clone with the highest titer, designated as producer cell line PA317/G1TkSvNa.53, was used to produce a master cell bank.

C. Preparation of pG1TK1SvNa

A schematic representation for producing pG1TK1SvNa shown in Figure 7, which was prepared to remove the partial open reading frame from pG1TKSvNa (Figure 6).

Generation of pSPTK5':

DNA from the plasmid pG1NaSvTk was digested with restriction enzymes BgIII and SmaI and the 1163 base pair (bp) Herpes thymidine kinase (TK) fragment was fractionated by agarose gel electrophoresis and isolated. This fragment contains 56 bp of the TK 5'-untranslated region and 1107 bp of the TK translation open reading frame. The 1163 bp TK fragment was ligated to the plasmid vector pSP73 (Promega Corporation, Madison, WI) that had been digested with restriction enzymes BgIII and SmaI. The resulting ligated plasmid construct was named pSPTK5' because it contains the 5' portion of the TK open reading frame but lacks the last 21 bp of the open reading frame and the translation termination codon.

PCR of the RK open reading frame:

pG1NaSvTK plasmid DNA was linearized by digesting it with BgIII. The linearized pG1NaSvTK was used as a template for polymerase chain reaction (PCR) using a forward primer that contains the first 17 bases of the TK open reading frame (5'-GCACCATGGCTTCGTACCCCTGC-3') and a reverse primer that contains complementary sequence for an XhoI site, the TK translation termination codon, and the last 19 bp of the TK open reading frame (5'-CCTGCATCGATTCTCGAGTCAGTTAGCCTCCCCCATCTCC-3'). 30 cycles of PCR were performed as follows: 1 minute at 94°C and 2 minutes at 60°C with a final 7 minute extension cycle at 72°C. PCR products were fractionated on an agarose gel and the expected 1215 bp fragment that includes the full-length TK open reading frame was isolated. The isolated fragment was digested with restriction enzymes PstI and XhoI, digestion products were fractionated on an agarose gel, and the 420 bp fragment was isolated. This fragment extends from the PstI site at the nucleotides encoding amino acids 249-250 of the TK open reading frame through the XhoI site

immediately downstream of the TGA translation termination codon.

Generation of pSPTK1:

pSPTK5' was digested with PstI and the 3993 bp fragment that contains the pSP73 vector and the 5' portion of the TK open reading frame was isolated following agarose gel electophoresis. This 3993 bp fragment was ligated to the PCR-generated 420 bp PstI/XhoI fragment that contains the 3' end of the TK open reading frame (above). Ligated plasmid DNA was transformed into E. coli DK5α competent cells (Gibco/BRL, Gaithersburg, MD) and DNA from ampicillin-resistant colonies was screened by restriction enzyme digestion. Plasmids that appeared to contain the full-length TK open reading frame were termed pSPTK1. The DNA from several pSPTK1 clones was dideoxy sequenced in the region from the PstI site through the XhoI site (the region that was generated by PCR). pSPTK1 clone #4 was found to match the expected TK sequence in this region and was used for construction of pG1TK1SvNa.

Generation of pG1TK1SvNa:

pSPTK1 DNA was digested with BgIII and the 5' overhanging ends were repaired by incubation of the digested DNA with deoxy nucleotides and Klenow fragment of E. coli DNA polymerase I. The DNA was then digested with XhoI to generate a 1225 bp fragment that contains 56 bp of TK 5'-untranslated region and the full-length TK open reading frame. This blunt/XhoI fragment was ligated to pG1XSvNa DNA that had been digested with SnaBI and SalI.

To construct pG1XSvNa, the 1.2 kb SvNa fragment was excised from pSvNa (Part A above) with SalI and HindIII. This fragment was ligated to pG1 that had been digested with SalI and HindIII. The ligated plasmid was termed pG1XSvNa where the "X" denotes a multiple cloning region.

The product DNA from the pG1XSvNa and TK ligation was transformed in DH5α and DNA from ampicillin-resistant

colonies was screened as previously described. Plasmids that appeared to contain the TK fragment by diagnostic restriction enzyme digestion were termed pG1TK1SvNa. Clone #2 was dideoxy sequenced from the beginning of the 5'-LTR through the end of the 3'-LTR and was found to contain the intact TK open reading frame.

pG1TK1SvNa was used to produce producer cell lines by combination with PA 317 by the hereinabove described method (Part B above). The titers of the viral particles produced by the cell lines were examined. One high-titer producing cell line, designated as producer cell line PA 317/G1TK1SvNa.7, was chosen for clinical development.

D. Administration of Producer Cells to Human Patients

Eight patients with malignant brain tumors (six with recurrent glioblastoma, one with metastatic renal cell carcinoma, and one with metastatic malignant melanoma) were given injections of PA317/G1TkSvNa. 53 producer cells. In one patient, two tumors were treated.

Each patient was given multiple injections of the producer cells. The number and volumes of the intratumoral injections were determined by establishing the appropriate target volume based on the preoperative MRI scan. The gadolinium(Gd)-enhanced tumor mass was considered to represent one or more spheres (according to the configuration of the tumor). Each of these spheres then received 5 to 7 stereotaxic trajectories (creating columns of injected cells within the tumor mass) to deliver the producer cells in a homogeneous manner. Each injection was performed using a 100 μl Hamilton syringe and, depending on the total volume of the tumor and the available number of cells, each mm of tumor along the tract was injected with 50 - 100 μl of cell suspension ($5x10^6$ - 10^7 producer cells in Plasma-Lyte® A Injection, an electrolytic solution, pH 7.4, produced by Baxter Health Care Corporation , Deerfield, Illinois. The length of each column varied at

different areas of the tumor. Injections were initially performed via a single burr hole in the skull.

To optimize cell distribution, the technique was modified after the first two patients were injected. Cells were seen exuding along the injection tract and a concentration gradient was created due to the expanding trajectories from a single point origin of injection. Accordingly, a craniectomy (removal of skull) replaced a single burr hole, thus exposing a larger surface of the brain and enabling multiple injections at parallel trajectories.

Seven days after the cell injections, each patient was given ganciclovir (Cytovene, Syntex Corp.) intravenously, in a dose of 5 mg/kg body weight, daily for 14 days. MRI scans were performed frequently at the early stages of the treatment and at two to 4 weeks intervals thereafter. Positron emission tomography (PET) scans using radioactive glucose (FDG; fluoro deoxy glucose), were performed before and immediately after treatment in patients in whom PET identified a hypermetabolic tumor before treatment.

Five of the eight patients had evidence of antitumor response. This was characterized by a decrease in tumor size (in three patients) and/or a change in the tumor's consistency (micro and macrocysts, reduced gadolinium enhancement, in five patients).

After the initial response, a transient increase in the region of gadolinium enhancement occurred in two patients two weeks after finishing treatment. Enhancement of the injection tracts also was evident. This reversed spontaneously two to three weeks later (four to five weeks after completion of treatment) and may represent a transient, self-limiting inflammatory response. Multiple biopsies taken from one of these patients confirmed the presence of a diffuse inflammatory response with tumor

cell necrosis. Small islands of scattered tumor cells, however, persisted in the region of treatment, and the patient received a second course of treatment. Th injection of the producer cells and the ganciclovir treatment were tolerated very well by all patients.

Since the volumes of the treated tumors and the available number of producer cells varied greatly, a dose response curve was plotted for the concentration of cells per unit volume of tumor (in cc) and the antitumor response. Tumor volumes were measured using a commercially-available computerized image analysis of MRI scans. Antitumor response was measured by obtaining the ratio between tumor volume at one and two months divided by the tumor volume before treatment. A strong correlation was found between the number of injected cells (the DOSE) and antitumor response (EFFECT) (Figure 8). It is also evident from that plot that a minimum of 2.5×10^8 producer cells are needed for every cc of tumor to effect a persistent antitumor effect. Lower concentrations were associated with either transient antitumor effect or no effect at all.

Example 2

Gene Therapy for the Treatment of Brain Tumors Using an Implantable Reservoir

A craniotomy is performed and a frozen section is taken to confirm viable glioblastoma cells and optimal tumor removal is attempted. The patient's tumor is cryopreserved. The surgical margin of the cavity is infiltrated at multiple sites with the G1Tk1SvNa.7 vector producer cells (day 0) to a maximum volume of 10 ml. Vector producer cells suspended in Plasma Lyte A at a concentration of 1-2 times 10^8 cells/ml are inoculated slowly in 0.25 to 0.5 ml inocula at sites distributed as evenly around the tumor sites as possible. An Ommaya reservoir is placed into the tumor bed to allow future

access to this area. A diagram of the sites of inoculation and volumes of cells delivered at each site is kept as part of the permanent research records. An MR scan is performed within 48 hours following surgery to define the size of the residual tumor.

Seven days after the initial surgery, the Ommaya reservoir has sealed into the patient's brain and surrounding tissues. On day 7, skin overlying the Ommaya reservoir is cleansed with betadine. A needle is inserted through the overlying skin into the reservoir with the patient awake. With the patient in the recumbent position, the Ommaya reservoir is gently irrigated with normal saline to clarify the contents of the tumor cavity and to confirm the estimate of tumor cavity volume.

Additional vector producer cells in suspension are delivered following the attempted removal of an equivalent amount of fluid from the tumor cavity. Preferably, no less than 5 ml and no more than 10 ml of the suspension is delivered. If fluid is obtainable from the Ommaya catheter at this time, a 1 ml sample is frozen and archived for possible further testing. If fluid cannot initially be withdrawn from the Ommaya catheter, the vector producer cell suspension will be injected over not less than one hour. The administration of cells through the Ommaya reservoir is followed by a flush of normal saline equal in volume to the volume of the Ommaya delivery system.

Ganciclovir is administered twice a day by IV infusion over one hour (dose of 5 mg/kg) starting on the 21st post-operative day for 14 days (days 21 to 34), followed by a 7-day rest period. This completes the initial treatment cycle.

Patients may receive additional treatment cycles, preferably up to five, depending on the anti-tumor response, toxicities, and the patient's clinical status. MR scans are done at the end of each cycle. Only patients

with responding or stable disease will continue treatment. If assessment of the MR scan leaves an uncertainty as to whether an increased size of an enhancing lesion is due to tumor progression or inflammatory changes, a biopsy is taken to make a definitive diagnosis. Each repeat cycle consists of vector producer cells injected through the Ommaya reservoir on day 0 of the retreatment cycle. Ganciclovir treatment commences on day 14 and continues for 14 days (days 14 to 27). No treatment is given for 7 days (days 28 to 34). Upon completion of the patient's last cycle of ganciclovir, the patient is followed at post-treatment months 1, 2, 3, 5, 7, 9, and 12, then every three months for the second year, and then at least annually for life.

Factors such as tumor size, location and the pre-operative neurological condition of the patient will determine the injectable volume. The volume of injected cells is, preferably, not in excess of 10 ml. The final cell concentration will be adjusted to 1-2 times 10^8 cells/ml. All patients receive a single dose of an antibiotic, such as Vancomycin, just prior to the initial surgical procedure and prior to the insertion of the Ommaya reservoir. All patients may also receive Dexamethasone starting 7 days prior to the initial surgery. Following the surgery, the dose will be tapered based upon the clinical condition of the patient in order to wean the patient off Dexamethasone treatment. Patients may receive mannitol during the surgical procedure at 1 g/kg, and the dose may be repeated t.i.d. following the procedure, as clinically indicated. Anticonvulsant therapy is administered according to the usual neurosurgical guidelines. Pain medications may include acetaminophen, preferably in a dosage of 650 to 1000 mg q 4 hours. Patients may also receive G-CSF support for neutropenia (less than 500 cells/mm^3).

The disclosures of all patents, publications (including published patent applications), and database entries referenced in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry was specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of treating a tumor in a human patient, comprising:

transducing tumor cells in vivo with a nucleic acid sequence encoding an agent which is capable of providing for the inhibition, prevention, or destruction of the growth of said tumor cells upon expression of said nucleic acid sequence encoding said agent.

2. The method of claim 1 wherein said transducing of tumor cells in vivo with a nucleic acid sequence encoding an agent which is capable of providing for the inhibition, prevention, or destruction of the growth of said tumor cells comprises:

administering to said tumor producer cells including said nucleic acid sequence encoding said agent, whereby said producer cells generate viral particles including said nucleic acid sequence encoding said agent, and which are capable of transducing said tumor cells.

3. The method of claim 2 wherein said agent is a negative selective marker, and further comprising administering to said human patient an interaction agent which interacts with said negative selective marker in order to inhibit, prevent, or destroy the growth of said tumor cells.

4. The method of claim 1 wherein the tumor is a brain tumor.

5. The method of claim 4 further comprising the steps of:

(a) administering viral producer cells into said brain tumor at a plurality of sites, said sites being positioned in said tumor to effect a distribution of said cells through said tumor, wherein viral particles produced by said producer cells are effective to transduce cells of said tumor and said viral particles include a nucleic acid

sequence encoding an agent such that said tumor cells are rendered sensitive to an interaction agent; and thereafter

(b) treating said tumor with said interaction agent.

6. The method of claim 4 wherein said transducing step comprises:

(i) inserting a reservoir into the brain at the site of the tumor; and

(ii) adding viral producer cells to the reservoir.

7. The method of Claim 5 wherein the producer cells are stereotaxically administered.

8. The method according to claim 5, wherein step (a) comprises:

(i) obtaining a first image of said tumor in said patient;

(ii) from said first image, determining a plurality of parallel, stereotaxically-defined trajectories for microinjecting viral producer cells into said tumor, each trajectory of said plurality intersecting at least one deposition site for said cells, such that the totality of deposition sites are in a pattern that effects a homogeneous distribution of said cells throughout said tumor;

(iii) performing a craniotomy to expose the brain of said patient to accommodate microinjection along each trajectory of said plurality;

(iv) introducing a microinjection needle into said brain along each trajectory; and

(v) depositing said cells at each of said sites along such trajectory.

9. A method according to claim 8, wherein said virus is a retrovirus which comprises a tk gene operably linked to a promoter for expression in said tumor cells, wherein tumor cells transduced with said retrovirus are sensitive to the lethal effect of ganciclovir.

10. A method according to claim 8 wherein said obtaining of a first image comprises generating a CT, MR, PE or X-ray image of said tumor.

11. A method according to claim 10, wherein said first image is a gadolinium-enhanced MR image.

12. The method of Claim 5 wherein said agent is a negative selective marker.

13. The method of Claim 12 wherein said negative selective marker is selected from the group consisting of Herpes Simplex Virus thymidine kinase; cytomegalovirus thymidine kinase. varicella-zoster virus thymidine kinase; and cytosine deaminase.

14. The method of Claim 13 wherein said interaction agent is selected from the group consisting of ganciclovir, acyclovir, and 1-2-deoxy-2-fluoro-β-D-arabinofuranosil-5-iodouracil.

15. The method of Claim 12 wherein said interaction agent is ganciclovir.

16. The method of Claim 12 wherein said negative selective marker is Herpes Simplex Virus thymidine kinase.

17. The method of Claim 5 wherein said producer cells contain a retroviral vector.

18. The method of Claim 5 wherein said producer cells are administered in an amount of at least 2.5×10^8 per cc of tumor cells.

19. The method of Claim 18 wherein said interaction agent is administered in an amount of from 5 mg to 10 mg per kg of body weight.

20. The method of Claim 19 wherein the interaction agent is ganciclovir, and said ganciclovir is administered systemically.

21. The method of Claim 20 wherein the ganciclovir is administered intravenously.

22. The method of Claim 21 wherein the ganciclovir is administered in a dose of about 5 mg/kg of body weight.

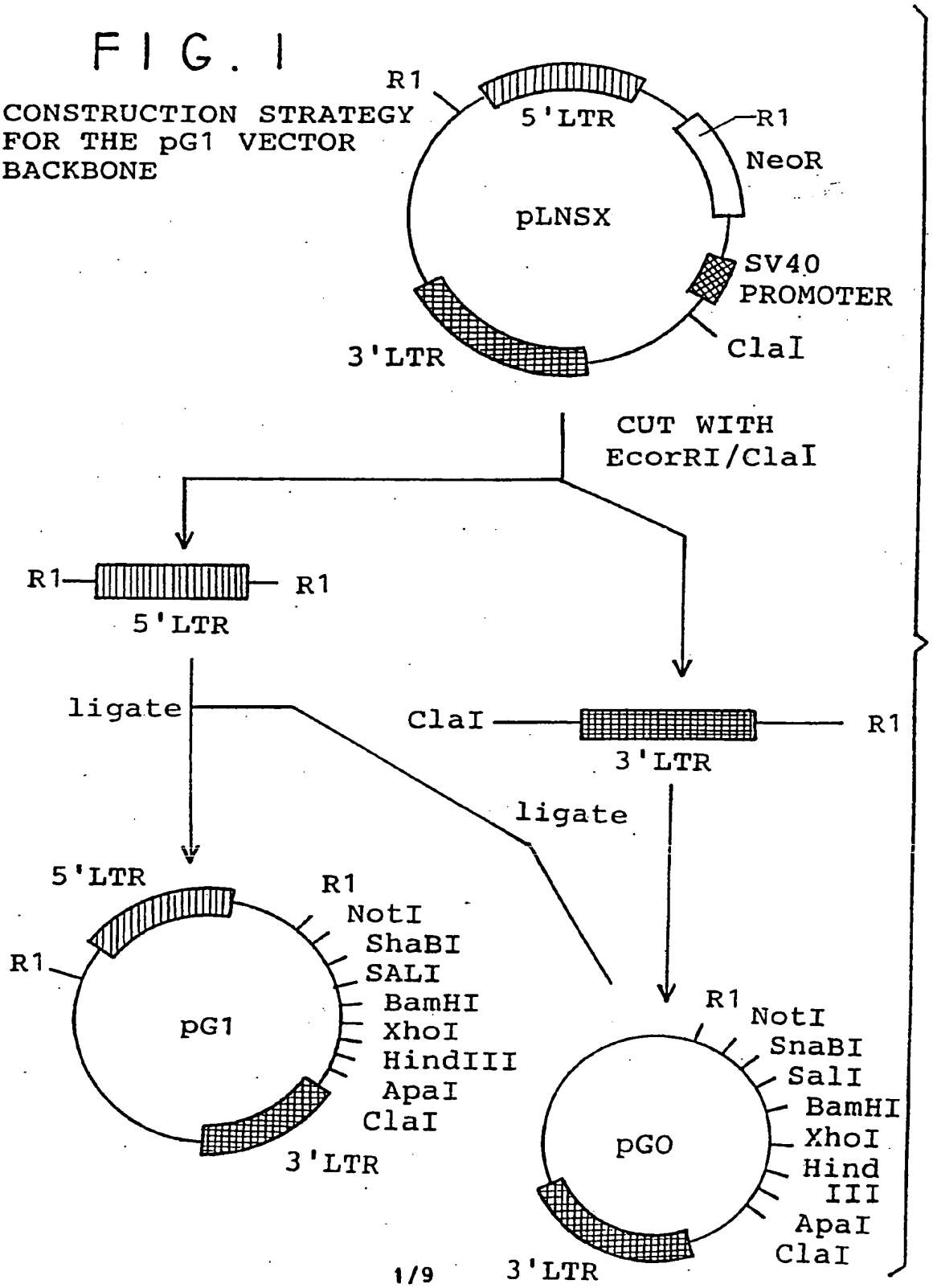
23. A product for treating a tumor in a human host comprising a viral producer cell which produces a viral particles which includes a nucleic acid sequence encoding an agent such that said tumor is rendered sensitive to an interaction agent.

24. The product of claim 23 wherein the product is for treating a human brain tumor.

25. The product of claim 24 wherein the product is used by injection into the brain tumor.

FIG. 1

CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE



SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

1/2 EcoRI	NotI	SnaBI	SalI	BamHI	XhoI	HindIII	ApaI
AATTC	GCGGCCGC	TACGTA	GTCGTC	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	CAGCTG	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2ClaI

AT

TAGC

FIG. 2

FIG. 3

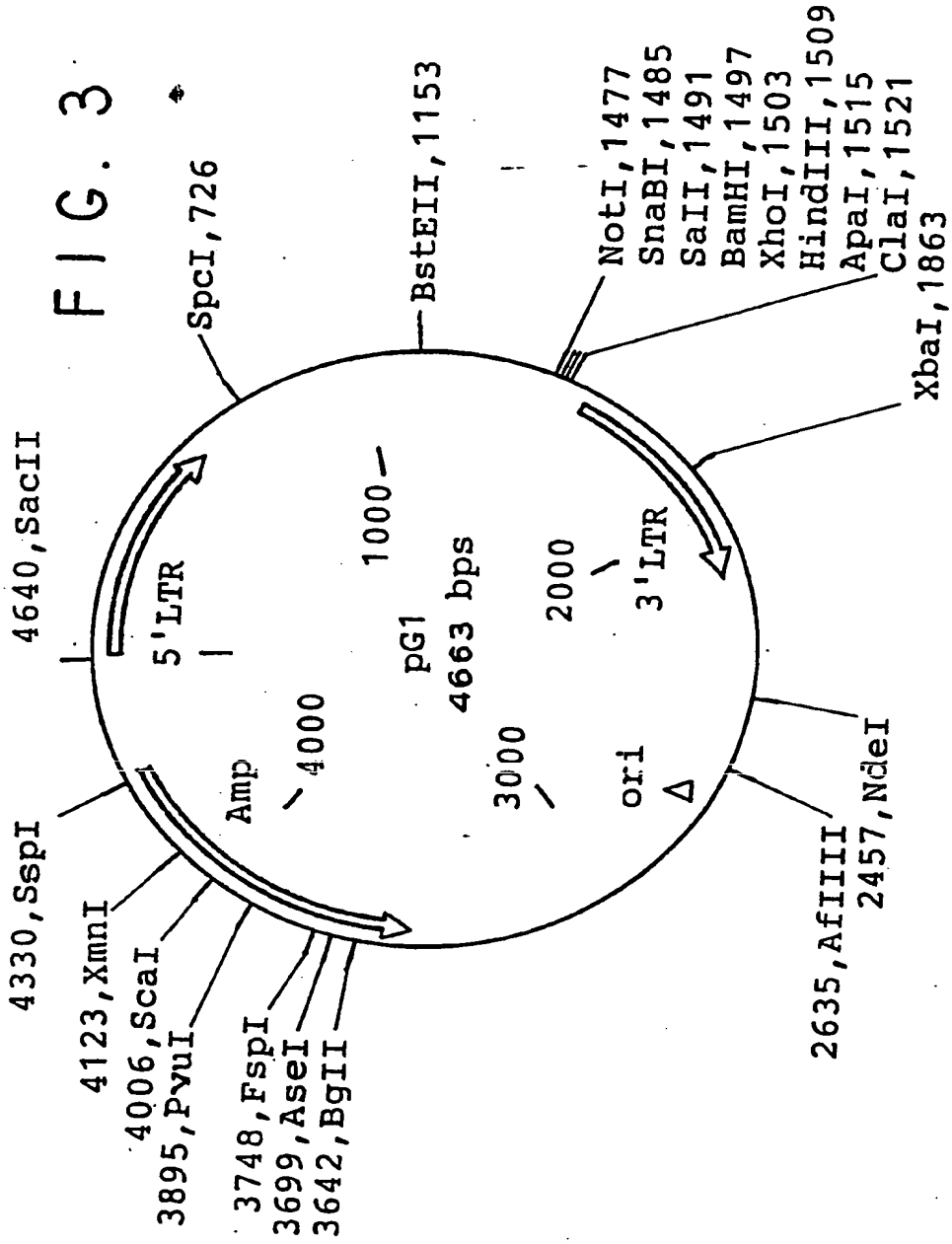
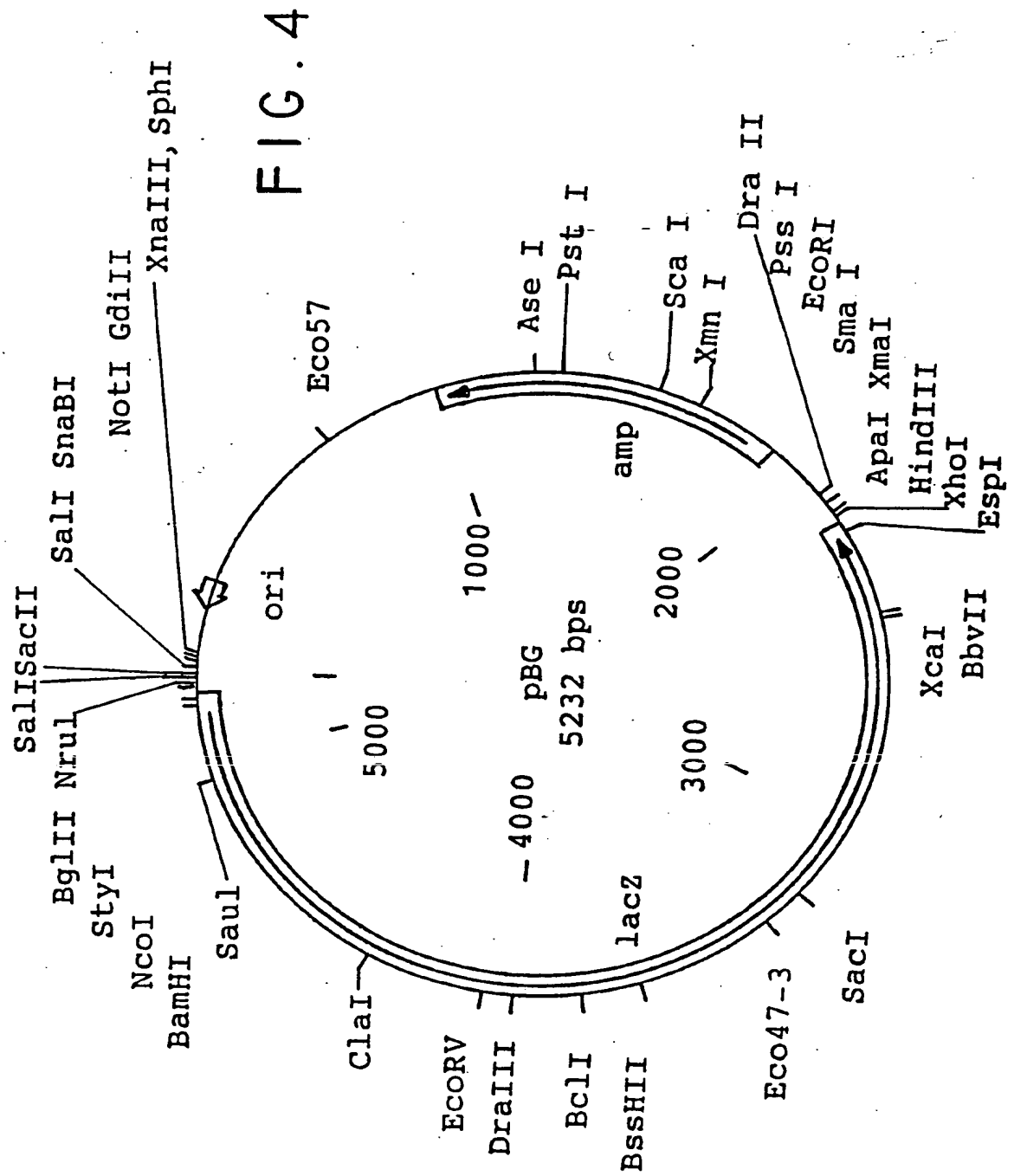

SpcI,726
BstEII,1153
NotI,1477
SnaBI,1485
SalI,1491
BamHI,1497
XhoI,1503
HindIII,1509
ApaI,1515
ClaI,1521
XbaI,1863
4640,SacII
5'LTR
1000
pG1
4663 bps
2000
3'LTR
Amp
4000
3000
ori
Δ
4330,SspI
4123,XmnI
4006,ScaI
3895,PvuI
3748,FspI
3699,AseI
3642,BglI
2635,AflIII
2457,NdeI

FIG. 4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5232 bps
1000
2000
3000
4000
5000
ori
amp
lacZ
Eco57
Ase I
Pst I
Sca I
Xmn I
Dra II
Pss I
EcoRI
Sma I
ApaI XmaI
HindIII
XhoI
EspI
XcaI
BbvII
SacI
Eco47-3
BssHII
BclI
DraIII
EcoRV
ClaI
SauI
BamHI
NcoI
StyI
BglII NruI
SalISacII
SalI SnaBI
NotI GdiII
XnaIII, SphI

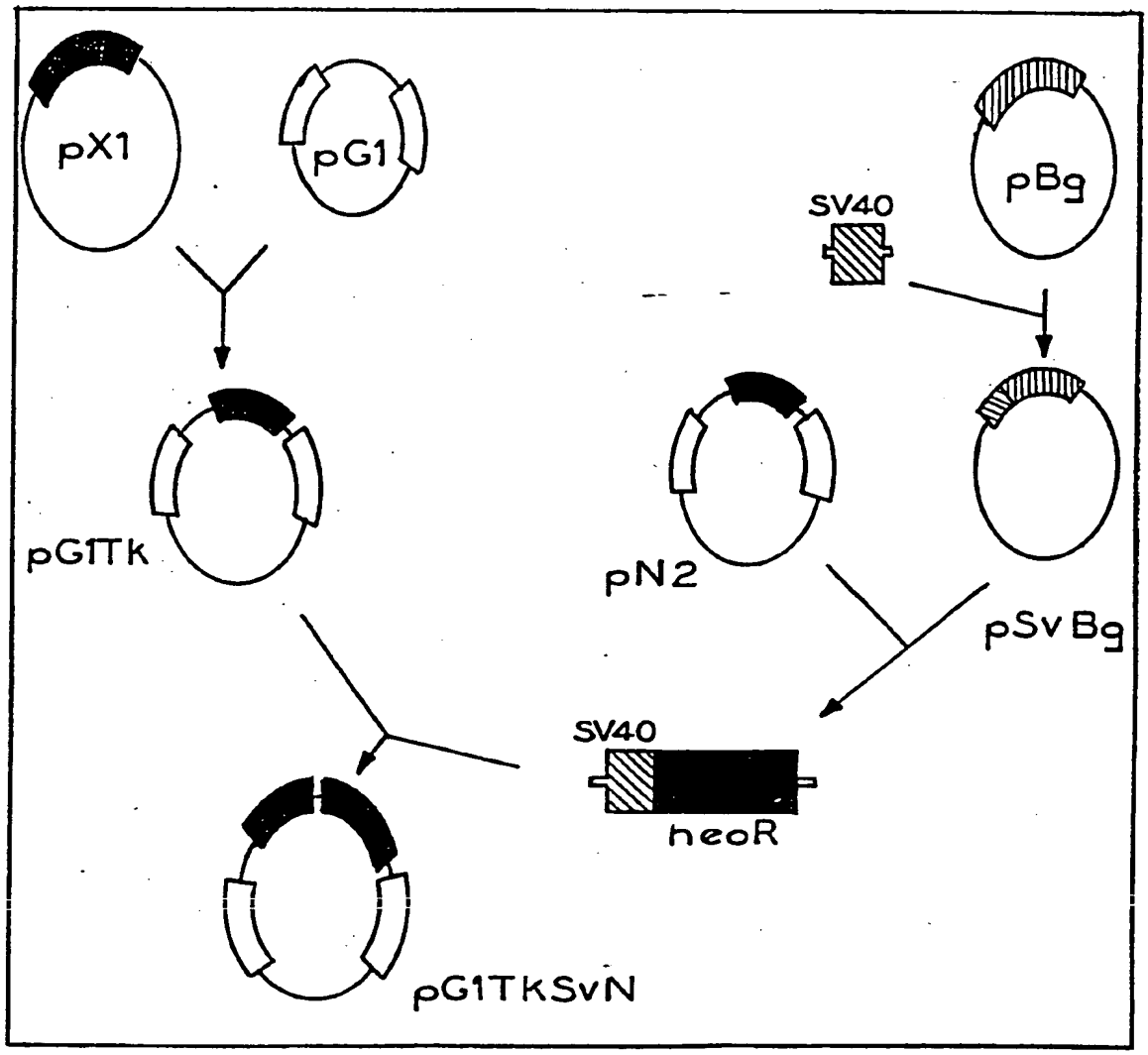
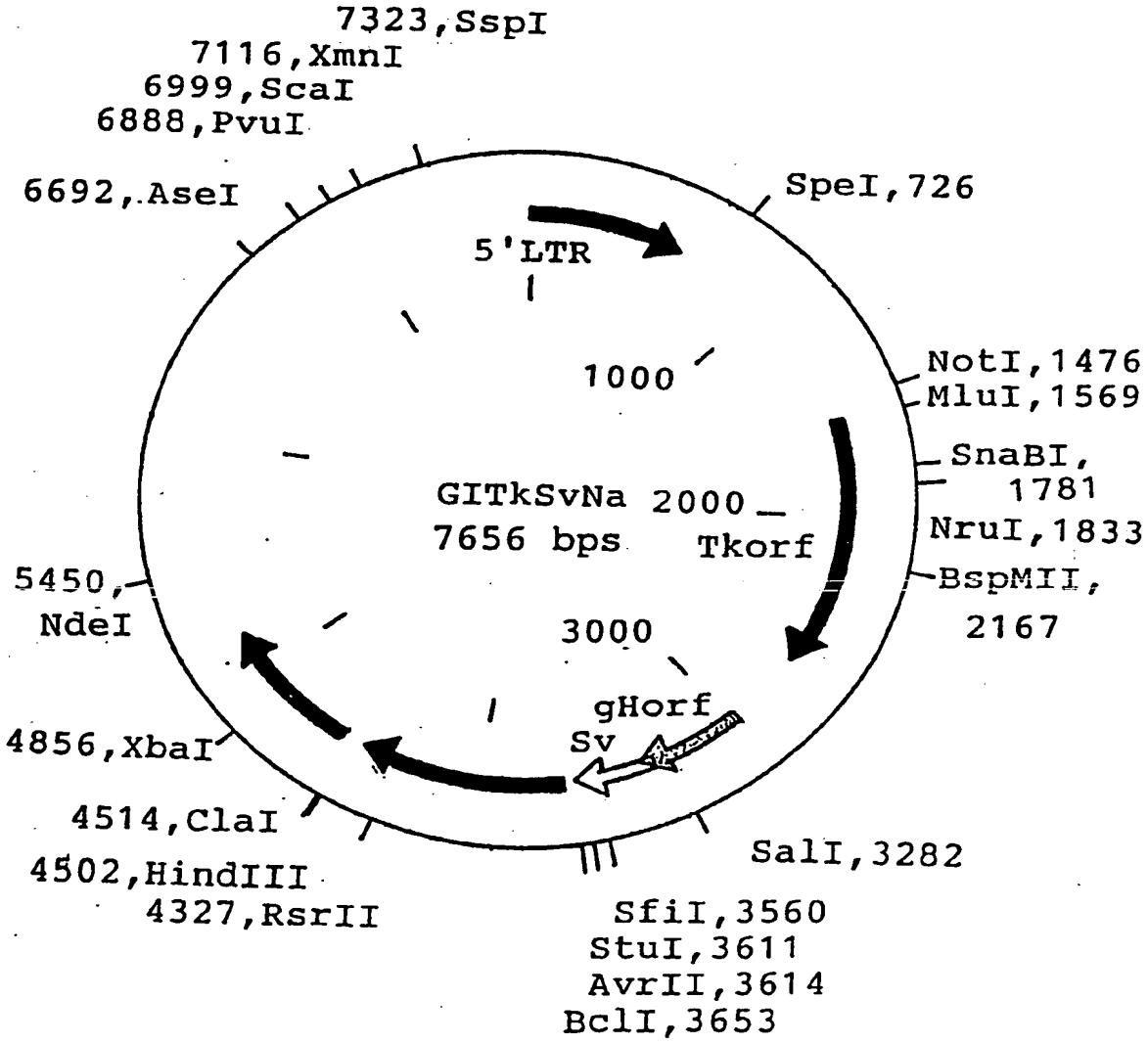


FIG. 5

FIG. 6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7116,XmnI
6999,ScaI
6888,PvuI
6692,.AseI
SpeI,726
5'LTR
1000
NotI,1476
MluI,1569
SnaBI,
1781
NruI,1833
BspMII,
2167
GITkSvNa
7656 bps
2000
Tkorf
5450,
NdeI
3000
gHorf
Sv
4856,XbaI
4514,ClaI
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SfiI,3560
StuI,3611
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BclI,3653

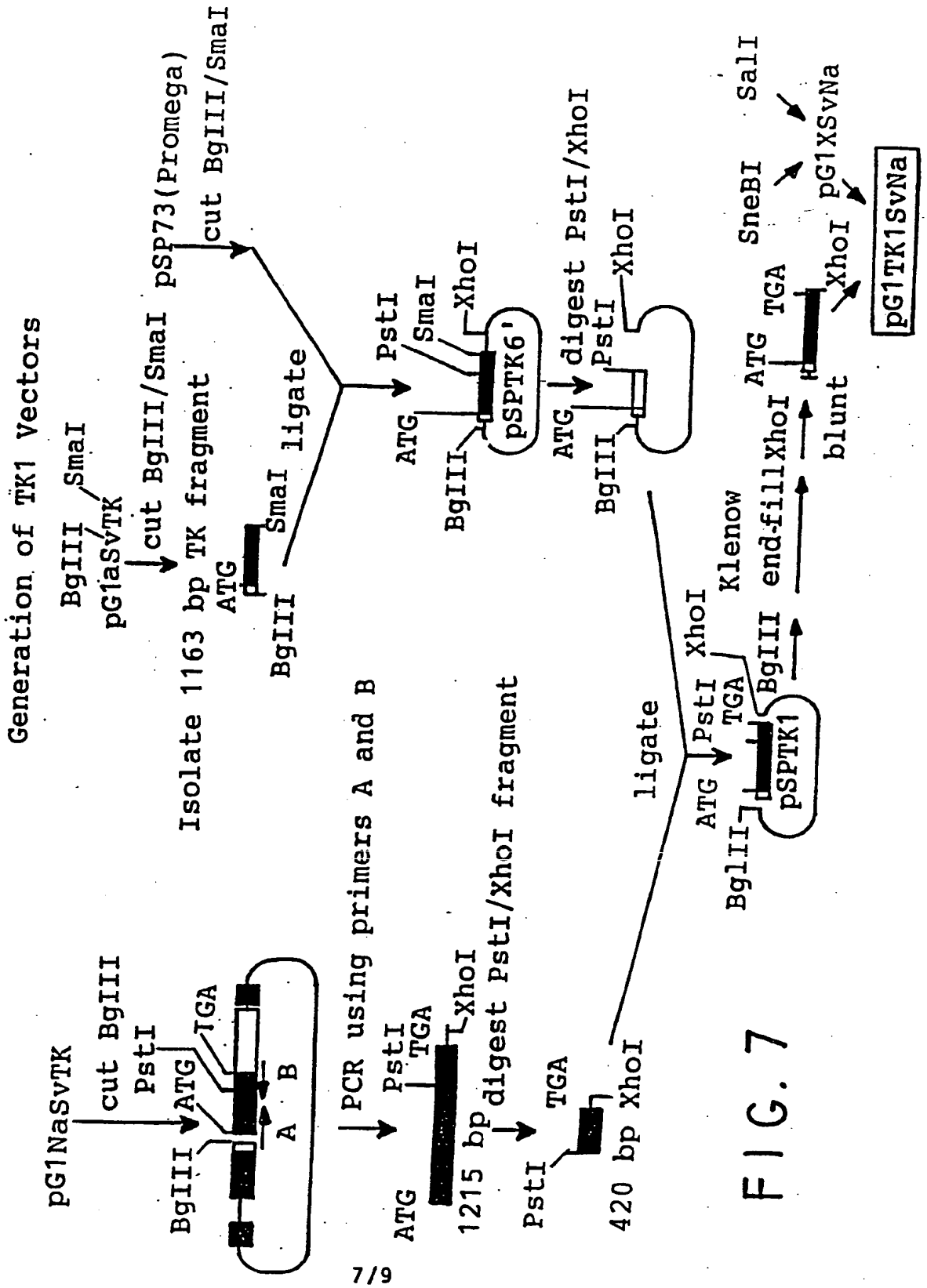

Generation of TK1 Vectors
pG1NaSvTK
cut BglII
PstI
ATG
TGA
BglII
A
B
PCR using primers A and B
PstI
TGA
XhoI
ATG
1215 bp
digest PstI/XhoI fragment
PstI
TGA
420 bp XhoI
ligate
PstI XhoI
Klenow
BglII
end-fill XhoI
blunt
pSPTK1
BglII
ATG
TGA
SneBI
SalI
XhoI
pG1XSvNa
pG1TK1SvNa
BglII SmaI
pG1aSvTK
cut BglII/SmaI
Isolate 1163 bp TK fragment
ATG
BglII
SmaI
ligate
pSP73(Promega)
cut BglII/SmaI
PstI
SmaI
XhoI
ATG
BglII
pSPTK6'
digest PstI/XhoI
PstI
XhoI
ATG
BglII

FIG. 7

FIG. 8

Correlation between the Concentration of Producer Cells/Tumor Volume and Response to Therapy

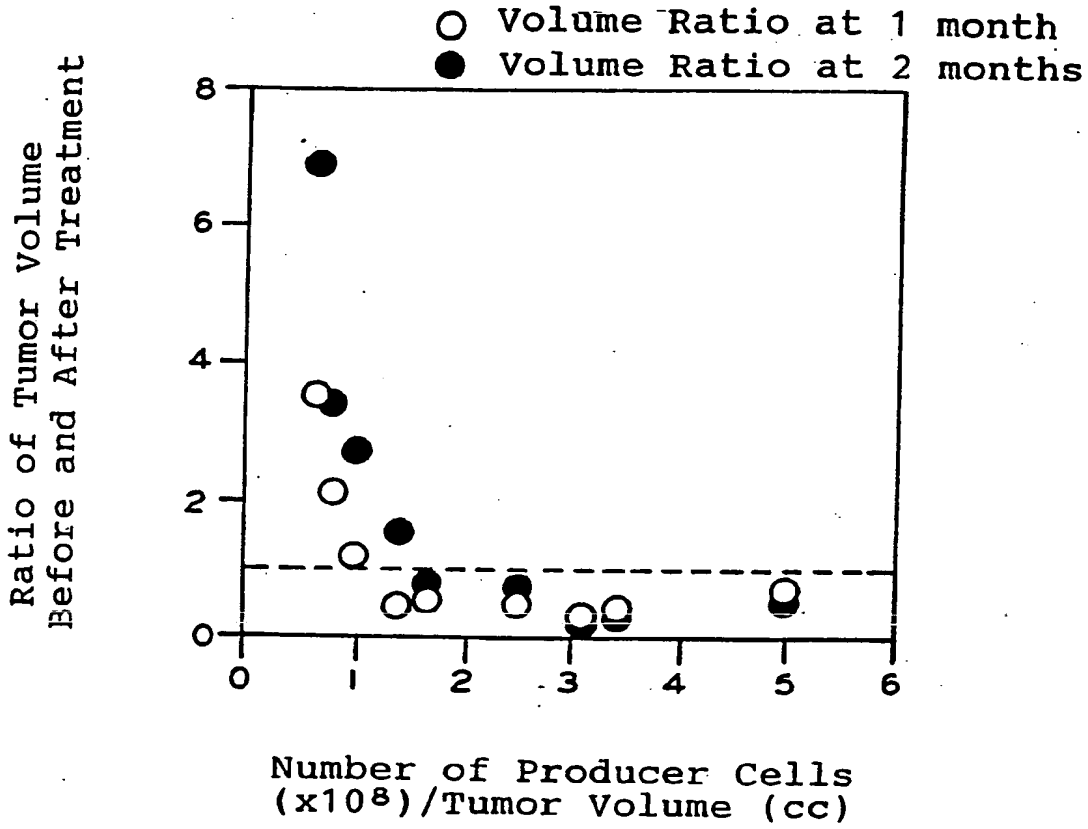
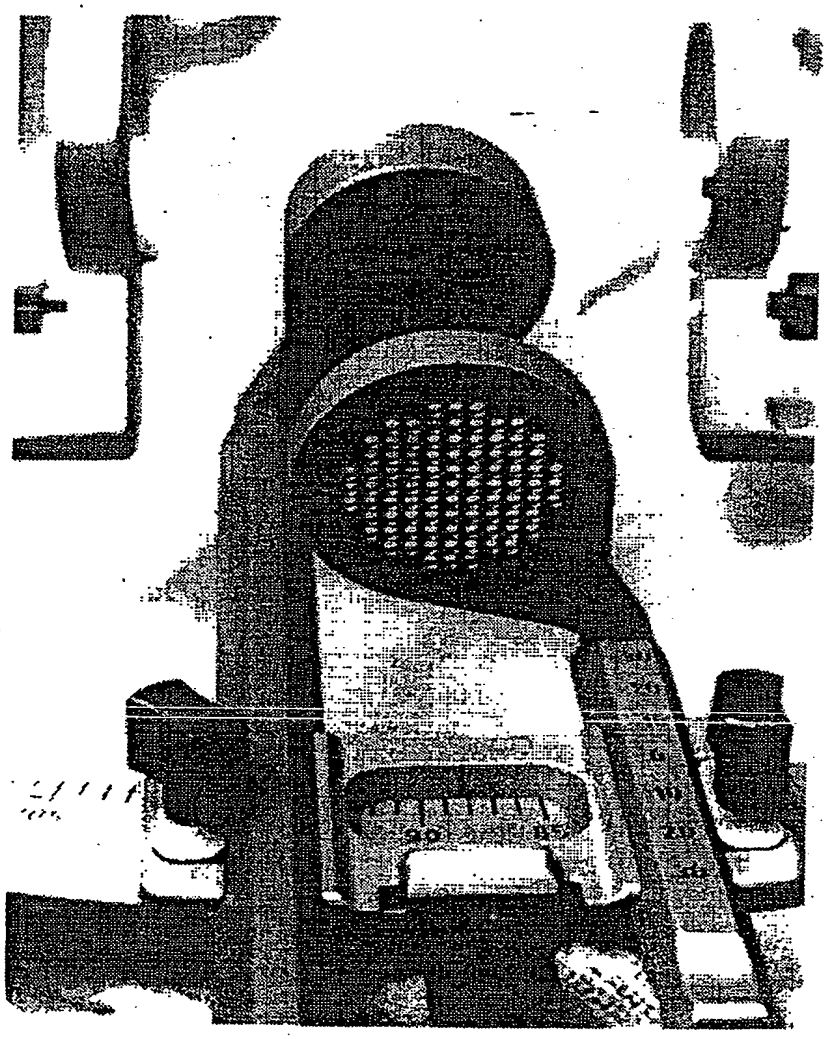


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09961

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 435/172.3, 320.1, 240.2, 935/62; 128/653.2, 659; 604/28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, WPIDS, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Proc. Natl. Acad. Sci. USA, Vol. 85, issued October 1988, Borrelli et al., "Targeting of an Inducible Toxic Phenotype in Animal Cells", pages 7572-7576, see entire document.	23-25 --- 1-22
Y	Journal of Cellular Biochemistry, Supp. 16F, issued 3-16 April 1992, Freeman et al., "Tumor Regression When a Fraction of the Tumor Mass Contains the HSV-TK Gene", page 47, Abstract V 209, see entire abstract.	1-25
X --- Y	Journal of the National Cancer Institute, Vol. 82, No. 4, issued 21 February, 1990, Moolten et al., "Curability of Tumors Bearing Herpes Thymidine Kinase Genes Transferred by Retroviral Vectors", pages 297-300, see entire document.	1-3, 23-25 --- 4-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 DECEMBER 1994	13 DEC 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JACQUELINE STONE Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09961

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,892,538 (AEBISCHER ET AL.) 09 January 1990, see entire document.	6-11
Y	US, A, 4,583,538 (ONIK ET AL.) 22 April 1986, see column 1, lines 33-51 and claims.	7-11
Y	US, A, 4,813,399 (GORDON) 21 March 1989, see column 4, line 66 and claim 1.	7-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09961

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 48/00, 51/00; C12N 15/86, 15/63, 15/38, 5/10, 5/08; A61B 5/055, 6/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.21; 435/172.3, 320.1, 240.2, 935/62; 128/653.2, 659; 604/28